ISOLATION AND MOLECULAR DETECTION OF BIOFILM PRODUCING AND MULTIPLE DRUG RESISTANT *Enterococcus faecalis* FROM THE BUFFALO MEAT

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ABSTRACT

The present study was conducted to determine the drug resistance pattern and the occurrence of biofilm producing *Enterococcus faecalis* from buffalo meat samples collected from in and around the Anand city, Gujarat. A total of 100 samples (meat, hand's swab, knife's swab) were collected aseptically from the butcher shops. Out of 100 samples, 52 (52%) samples were found to be positive on selective media, which were subjected to polymerase chain reaction revealing that 40 isolates were of *Enterococcus faecalis.* Antibiotic sensitivity test showed that all the isolates were sensitive to Ampicillin (100%); sensitive to intermediate for Gentamicin and resistance was observed against Tigecycline (85%), Trimethoprim (60%), Vancomycin (50%), Norfloxacin (37.5%), and Imipenem-cilastatin (25%). Out of all the PCR positive isolates, 95% (38/40) were biofilm producers when observed phenotypically on Congo Red Agar (CRA). So, it can be concluded that the buffalo meat can be a possible intermediary vehicle for the spread of multidrug-resistant biofilm producing enterococci strains to humans.

Keywords: *Bubalus bubalis*, buffaloes, antimicrobial resistance, biofilm, buffalo meat, carabeef, *ddlE* gene, *Enterococcus faecalis*, PCR, vancomycin resistant enterococci

INTRODUCTION

Enterococci are found all over the world. Many metazoans, from insects to humans, have enterococci as commensals in their gastrointestinal tracts. They are typically harmless in the intestine, but they can become deadly when they infect locations outside of the gut. In 1899, a case of infective endocarditis was the first disease linked to *Enterococcus* (Maccallum and Hastings, 1899). Enterococci are the third most common nosocomial pathogen, accounting for 12% of all hospital infections, and are responsible for urinary tract and wound infections, infective endocarditis, endophthalmitis, and peritonitis, all of which are frequently complicated by antibiotic drug resistance (Fischetti *et al*., 2006).

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Enterococcus faecalis can endure a heat of 60°C for 30 minutes in neutral medium, a pH range of 3.5 to 11, and a high salt tolerance (6.5% NaCl) (Fisher and Phillips, 2009). It can also handle minor stress and sloppy food preparation procedures. Because of their ability to live in extremely adverse conditions, they are useful as faecal contamination indicators (Wheeler *et al*., 2002). Antibiotic resistance is on the rise, and it is estimated that 10 million people will die worldwide as a result of resistant bacterium infection in the next thirty years (a larger figure than the 8.2 million deaths expected from cancer) (O'Neill, 2016). Antimicrobial misuse in human and veterinary medicine can result in the development of resistant strains (Sundsfjord *et al*., 2001). The advent of antimicrobial-resistant animal enterococci poses a significant danger of transfer of these bacteria to humans (Aarestrup *et al*., 2008; Hayes *et al*., 2003; Olsen *et al*., 2012). Difficulties in treating *Enterococcus faecalis* have arisen as a result of acquired resistance, notably multi-drug resistance enterococci, particularly vancomycin-resistant enterococci, which are a major source of concern for the medical community (Kročko *et al*., 2011).

Biofilm is a hydrated matrix of exopolymeric molecules, proteins, polysaccharides, and nucleic acids encasing a population of cells that are firmly adhered to various biotic and abiotic surfaces (Costerton and Stewart, 2001). According to the National Institutes of Health, biofilms are medically significant, accounting for more than 80% of microbial infections in the body. Biofilms are particularly difficult to remove from live hosts because bacteria in them are resistant to phagocytosis (Lewis, 2001). Biofilm development could have a significant impact since enterococci in biofilms are more resistant to drugs than planktonic enterococci.

With India being one of the largest exporters of carabeef in the world, ensuring safety of the meat is very important. The goal of this study was to look at antibiotic sensitivity and resistance pattern of *Enterococcus faecalis* isolated from buffalo meat samples, as well as the ability of enterococci to build biofilm.

MATERIALS AND METHODS

Collection and processing of samples

Total 100 samples (70 buffalo meat samples, 15 butcher's hand swabs. 15 butcher's knife swabs) were obtained from the shops in and around the Anand city (Gujarat, India). Samples were collected aseptically in pre-sterilized tubes. All the samples were collected with consent from the butchers in presence of a qualified veterinarian.

Isolation and identification of *Enterococcus faecalis* from the samples

As per the method described by Majhenič *et al*. (2005), the samples were subjected to enrichment in 5 mL of *Enterococcus* selective broth individually in sterile test tubes for 18 h at 37°C. The samples in which the Enterococci were present turned blue. The loopful of enriched culture from the positive tubes was inoculated on Citrate Azide Tween Carbonate (CATC) media and incubated at 37°C for 18 h. Growth on CATC was observed and the isolated bacteria were subjected to Gram's staining. Gram-positive diplococci were identified as *Enterococcus* spp.

Biochemical characterization of *Enterococcus* species isolates

The Biochemical tests like Absence of catalase, Hydrolysis of bile esculin, Growth

in the presence of 6.5% NaCl and Hydrolysis of PYR (L-pyrrolidonyl-ß-napthalamide) were performed as per standard procedures for further confirmation and biochemical characterization of *Enterococcus* species isolates as described by Manero and Blanch (1999).

Confirmation of the *Enterococcus faecalis* by PCR

Culturally and biochemically positive *Enterococcus* spp. isolates were subjected to molecular characterisation using PCR targeting the *ddlE* gene for confirmation. The snap chill process was used to produce template DNA from *Enterococcus* strains. The PCR was done with primers that targeted the Enterococci *ddlE* gene (Table 1). 25 µL reaction mixture consisted of template DNA 5.0 µL, Primers 2.0 µL (10 pmol each primer), Mastermix (2X) 12.5 µL and nuclease free water 5.5 µL. The PCR amplification was carried out in an automated thermal cycler, the protocol given in Table 2. The PCR products were analyzed by submarine electrophoresis in 1.5% agarose gel in Tris-Borate-EDTA (TBE) buffer (1x). The gel was visualized under a UV transilluminator, and the images were documented in a gel documentation system.

Determining sensitivity of organisms towards antibiotics

The test was performed as per the methods described by Kasimoglu-Dogru *et al.* (2010) 83 of 106 analyzed neck skin samples were positive for Enterococcus, with *E. faecium* as the most prevalent species (48%). Vancomycin (5 mcg), norfloxacin (10 mcg), gentamicin (30 mcg), ampicillin (2 mcg), trimethoprim (5 mcg), tigecycline (15 mcg), and imipenem-cilastatin (10 mcg) were among the antibiotics utilized. Antibiotics were chosen, and zone of inhibition was measured according to CLSI recommendations, and the isolates were classified as sensitive (S), intermediate (I) and resistant (R).

Evaluation of biofilm production by *Enterococcus* isolates

The isolates identified morphologically and molecularly by PCR were subjected to streaking on CRA under aseptic conditions as described by Sharvari and Chitra (2012). The plates were incubated at 37°C for 18 h.

RESULTS AND DISCUSSIONS

Fifty two of the 100 procured samples (45 meat samples, 3 hand swabs and 4 knife swabs) when enriched on selective broth revealed presence of *Enterococcus* spp. The samples in the tubes in which they were enriched turned blue (positive) indicating presence of *Enterococcus* spp. In the samples, and the tubes in which the broth colour remained unchanged were marked as Enterococci negative samples. Further on subjecting the loopful culture from the positive samples to selective media, *i.e.,* CATC agar and incubating it for 18 h at 37°C revealed the presence of Enterococci in all 52 samples (52%).

All the 52 isolates showing typical colony characteristics of *Enterococcus* spp., the gram-positive diplococci (Figure 1), were further processed for biochemical characterization. All the isolates were first streaked on Brain Heart Infusion agar and then further subjected to specific biochemical tests. All these isolates were catalase negative, produced black colonies on bile esculin medium, PYR positive, and produced visible turbidity in BHI broth with 6.5% NaCl, and were confirmed as typical *Enterococcus* spp. When

these isolates were subjected to Polymerase Chain Reaction (PCR) targeting *ddlE* gene (Figure 3), 40 isolates were confirmed to be of *Enterococcus faecalis*. Gomes *et al*. (2008) reported that enterococci are implicated in severe multi-resistant nosocomial infections. They reported, the prevalence of enterococci in selected Brazilian foodstuffs (raw and pasteurized milk, meat products, cheeses and vegetables investigated the prevalence of enterococci in a variety of Brazilian foods (raw and haracteriz milk, meat products, cheese and vegetables), for species identification, phenotypic haracterization and PCR technique were used. 52.5% of the 120 food items tested positive for enterococci, with meat and cheese being the most infected. At the species level, 263 isolates were identified from a total of 299 food enterococci isolates. There were 139 *E. faecium* isolates (46.5%), 80 *E. faecalis* isolates (26.8%), 36 *E. casseliflavus* isolates (12.0%), eight *E. gallinarum* isolates (2.7%), and 36 isolates (12.0%) that could not be identified with the primers employed. The results revealed higher prevalence of *E. faecalis* in the food samples as compared to the previous study.

Antibiotic sensitivity testing using antibiotic disc diffusion assay was done which showed that all the 40 isolates were sensitive to Ampicillin (100%); while resistance was observed against Tigecycline (85%), Trimethoprim (60%), Vancomycin (50%), Norfloxacin (37.5%) and Imipenem-cilastatin (25%). The isolates were sensitive to intermediate for Gentamicin. Hundred percent isolates showed multiple drug resistance phenotypes (Table 3, Figure 4).

Isolates confirmed with PCR were subjected to streaking on CRA, which revealed 95% (38/40) biofilm producers as they produced black colour colonies on CRA, whereas no change in colouration was observed in non-biofilm producing isolates (Figure 2). Esmaeili *et al*. (2018) used CRA to investigate biofilm development and virulence factors in Enterococci species isolated from clinical and normal flora samples from individuals (N = 147), finding that 38% of isolates formed black colonies on the medium (biofilm producer). Fallah *et al*. (2017) isolated 57 enterococcal isolates from urinary tract infection patients and examined them using standard microbiological techniques. The Modified CRA and Microtiter plate methods were used to examine the ability of biofilm formation among all positive isolates, revealing that 26.5% of *Enterococcus faecalis* isolates and 75% of *Enterococcus faecium* isolates were biofilm producers. No as such work has been carried out for isolation and molecular detection of *Enterococcus faecalis* from carabeef with special reference to biofilm production and multiple drug resistance. This work also emphasizes on importance of this organism and its role in spread to humans by consumption of contaminated carabeef.

Meagre studies of *Enterococcus* from carabeef disables string out discussion with previous studies. We anticipate this study may be the beacon for the other researchers of the similar fields.

CONCLUSIONS

Biofilms generated in food-processing environments are particularly important because they can operate as a persistent source of microbial contamination, resulting in food spoiling or disease transmission. It's possible that raw meat contains numerous key pathogenic microorganisms, posing a health concern. Although no incidences of foodborne enterococcal infections have been recorded, horizontal antimicrobial gene transfer is

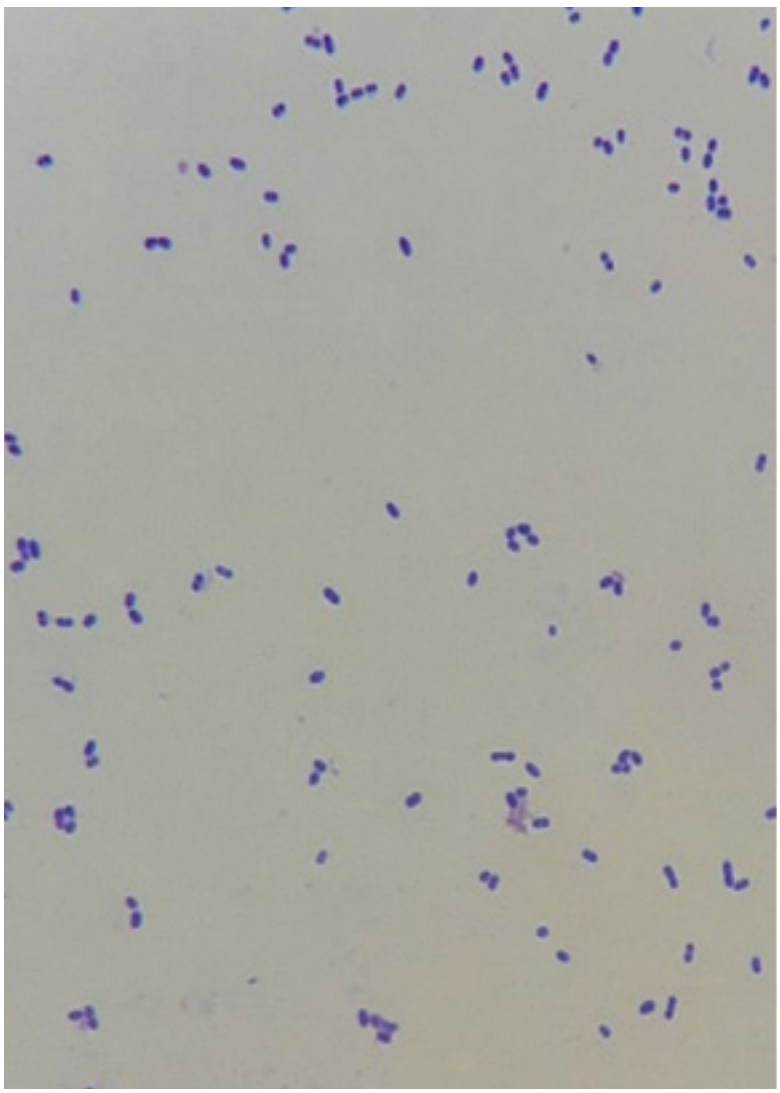

Figure 1. Gram positive diplococci.

Figure 2. Phenotypic characteristics of *E. faecalis* on Congo Red Agar.

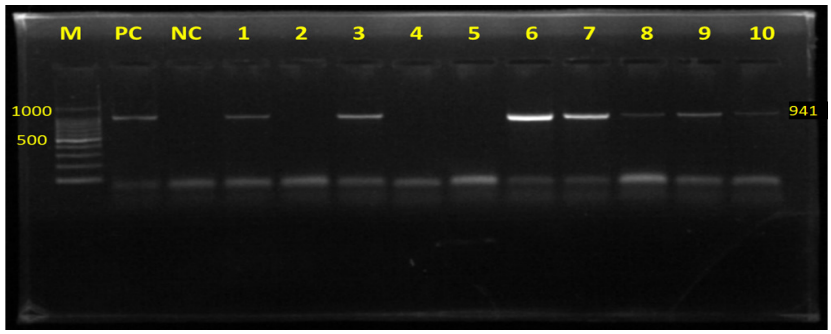

Figure 3. Polymerase chain reaction amplification of 941 bp *ddlE gene.*

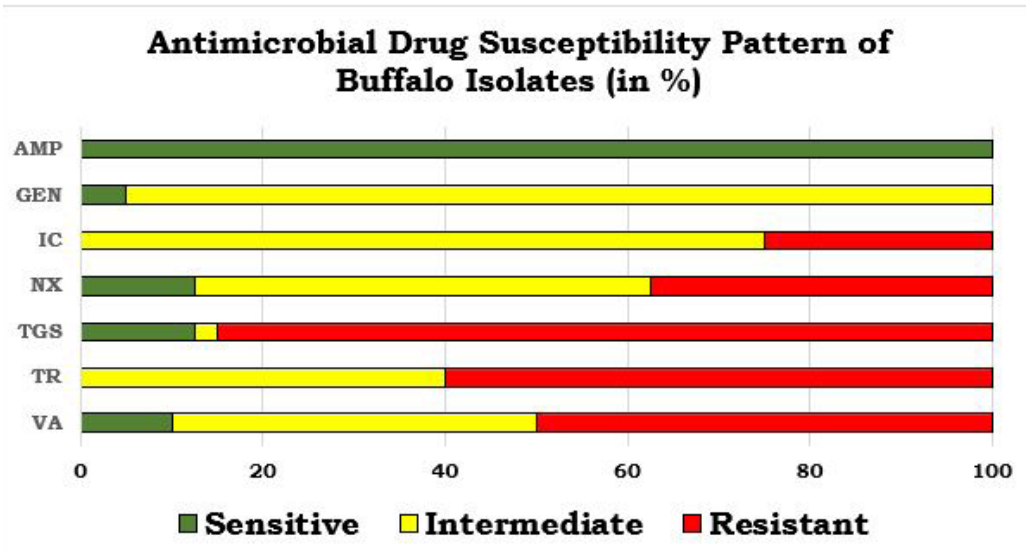

Figure 4. Antimicrobial susceptibility pattern of *E. faecalis* isolates.

Table 1. Description of primer used for detection of Enterococcus faecalis.

Cycling conditions		Temperature	Time
Initial denaturation		94ºC	10 minutes
30 cycles	Denaturation	94ºC	15 seconds
	Annealing	55ºC	15 seconds
	Extension	72ºC	45 seconds
Final extension		72ºC	5 minutes

Table 2. Master cycler conditions for ddlE gene.

Target gene	Primer sequence (5'-3')	Product size (Base pairs)	Reference
ddlE	F: ATCAAGTACAGTTAGTCTT	941	Dutka-Malen *et al*., 1995
	R: ACGATTCAAAGCTAACTG		

Table 3. Detection of multidrug resistance isolates.

Sr. No.	Antibiotics	Sensitivity (%)	Intermediate (%)	Resistance (%)
1	Ampicillin (AMP)	100	-	-
2	Tigecycline (TGS)	12.5	2.5	85
3	Trimethoprim (TR)	-	40	60
4	Vancomycin (VA)	10	40	50
5	Norfloxacin (NX)	12.5	50	37.5
6	Imipenem-cilastatin (IC)	-	75	25
7	Gentamicin (GEN)	5	95	-

a public health concern.

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